*De novo* transcriptomes built from hundreds of eye samples reveal hundreds of novel gene isoforms

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## Introduction

             The transcriptome is defined as the set of distinct RNA transcripts expressed in a population of identical cells. During transcription several RNA processing steps modify immature RNA and drive the formation of distinct isoforms for most genes. For example, the Gencode human transcript annotation (release 28) contains 97,713 protein coding transcripts across 20,306 genes (1). RNA processing broadly describes a variety of biological mechanisms and includes alternative promoter usage, alternative splicing, RNA editing, and alternative polyadenylation. Multiple studies have shown that gene isoforms can have distinct and critical functions in biological processes like development(2), cell differentiation(3), and cell migration(4). Alternative usage of isoforms has also been implicated in multiple diseases including cancer(5), cardiovascular disease (6), Alzheimer’s Disease(7) and diabetic retinopathy(8).

             Some of the first methods using short (~100bp) read RNA-seq to find novel gene isoforms focused on identifying novel exon-exon junctions and novel exon boundaries based on RNA-seq coverage. (9) More recently, several groups have developed specialized tools to use RNA-seq to reconstruct the whole transcriptome of a biological samples, dubbed *de novo* transcriptome construction (10),(3),(11).

*De novo* transcriptome construction uses short-read RNA-seq to reconstruct full-length mRNA transcripts. However, a large number of samples are necessary to overcome the noise and short-read lengths of this type of data. Because of increasingly inexpensive sequencing cost, datasets of the necessary size are now available. For example, one of the most comprehensive *de novo* transcriptome projects to date has been CHESS, which used the GTEx data set to construct *de novo* transcriptomes in over 9000 RNA-seq samples from 49 distinct location of the body to create a comprehensive annotation of mRNA transcripts across the human body. (12),(13) However, as the GTEx dataset lacks samples from any ocular tissues, the CHESS database is an incomplete annotation of the human transcriptome.

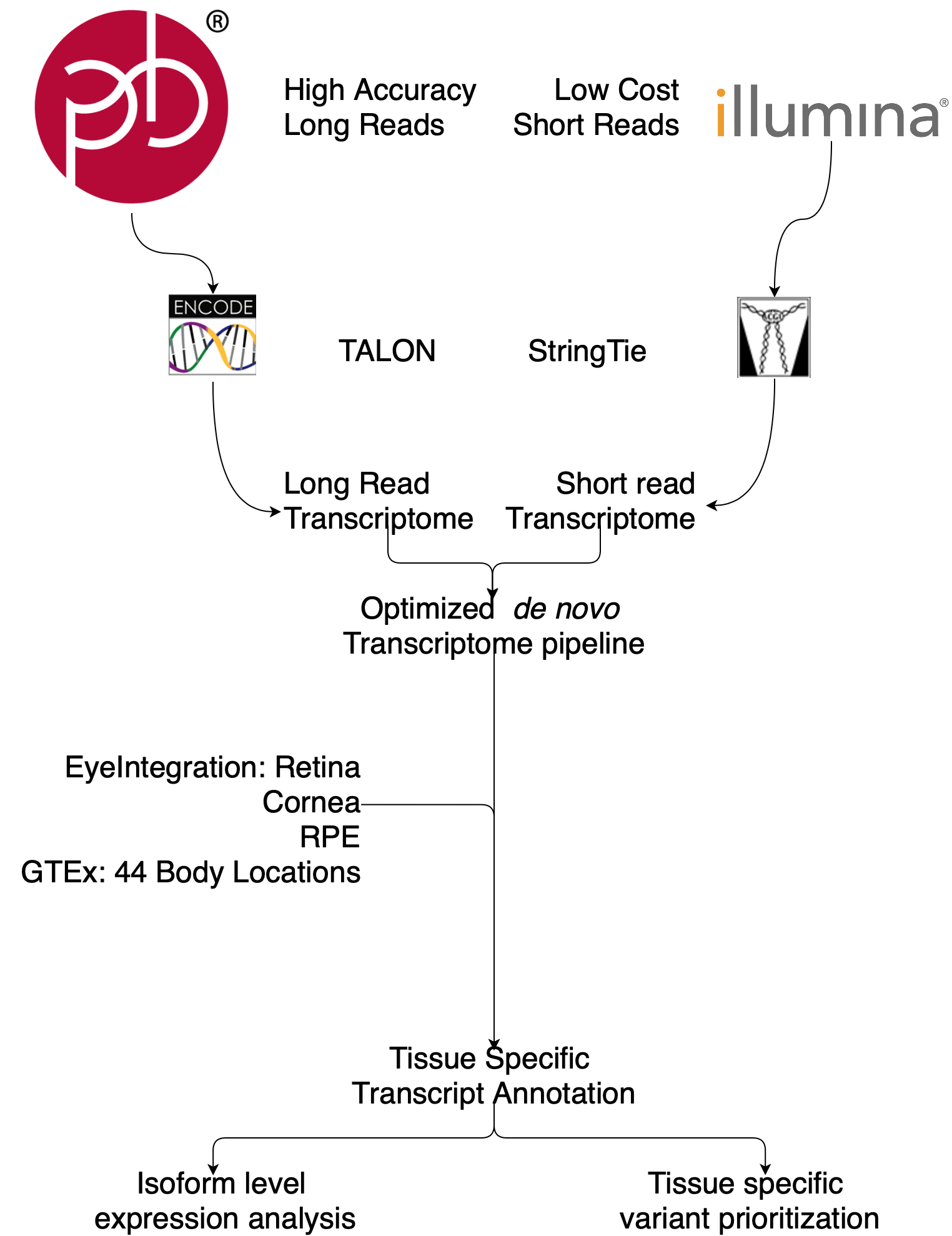
             Despite the increasing number of tools for transcriptome construction there has been no gold standard with which to evaluate the precision and sensitivity of *de novo* transcriptome construction on real (not simulated) biological data. Long-read sequencing technologies provide a potential solution to this problem as long-read sequencing can capture full length transcripts and thus can be used to identify a fuller range of gene isoforms. While previous iterations of long-read sequencing technologies typically had higher error rates, the new PacBio Sequel II system sequences long-reads as accurately as short-read based sequencing (14).

             We propose that long-read based transcriptomes can serve as a ground truth for evaluating short-read based transcriptomes; in this study we use PacBio long-read RNA sequencing to inform the construction of short-read transcriptomes. We generated PacBio long-read RNA-seq data from an induced pluripotent stem cell differentiated retinal pigmented epithelium (RPE) cell line along with matched Illumina short-read RNA-seq. Using the two sources of RNA-seq data we design a rigorous StringTie-based *de novo* transcriptome pipeline that maximizes the concordance between short and long-read *de novo* transcriptomes.

             We apply this pipeline to a previously published data set containing 368 ocular tissue samples compiled from mining publicly available short-read RNA-seq data (15). We use this pipeline to build transcriptomes in three major ocular subtissues: The cornea, retina, and the retinal pigmented epithelium (RPE), using RNA-seq data from both adult and fetal tissues to create a high-quality pan-eye transcriptome. In addition to our ocular samples, we used a subset of the GTEx data set to construct transcriptomes for 49 other locations across the body to facilitate comparisons among transcriptomes across the body.

             We use our gold-standard informed pan-eye de novo transcriptomes to reveal hundreds of novel gene isoforms in the eye and analyze their potential impact on ocular biology and disease. We provide our *de novo* transcriptomes as a resource to other researchers through an R package

## Methods



(Supplemental) Figure 1

Supplemental Figure 1. Workflow for De novo Transcriptome construction and analysis.(\*\*check to see if allowed to use pacbio logo)

## Generation of PacBio long-read RNA sequencing data and Illumina short-read RNA sequencing

             Human induced pluripotent stem cells (iPSCs) were differentiated into RPE using previously described protocols in (16) and (17). iPSC-derived RPE cells at 42 days post differentiation were lysed with TRIzol reagent (Thermo Fisher Scientific; cat # 15596026) and total RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). Total RNA samples that passed quality control metric (RIN >.9) were used for library preparation and sequenced as … on Illumina … and PacBio instruments at the National Institutes of Health Intramural Sequencing Center (NISC).

## Code availability and software versions.

             To improve reproducibility, all code used to generate both the data and figures for this paper was written as multiple Snakemake pipelines. (18) All code (and versions) used for this project is publicly available in the following github repositories: <https://github.com/vinay-swamy/ocular_transcriptomes_pipeline> (main pipeline), <https://github.com/vinay-swamy/ocular_transcriptomes_longread_analysis> (long-read analysis pipeline), <https://github.com/vinay-swamy/ocular_transcriptomes_paper> (figures and tables for this paper), <https://github.com/vinay-swamy/ocular_transcriptomes_shiny> (webapp)

WE ALSO NEED TO GIVE A GIT TAG FOR EACH TO INDICATE THE “RELEASE” VERSION WITH THIS PAPER (ALSO DEPOSIT IN ZENODO).

             All computational analyses performed in this project are run using multiple Snakemake workflows. Each Snakefile contains the exact parameters for all tools and scripts used in each analysis. All Snakefiles are included as supplementary data.(supplementary data files 1-4)

## Analysis of long-read data

             PacBio hifi reads were processed into full length, non-chimeric (FLNC) reads using the IsoSeq3 3.1.2 pipeline in the Pacbio SMRT link v7.0 software. The existing ENCODE long-read RNA-seq pipeline (<https://github.com/ENCODE-DCC/long-read-rna-pipeline>) was rewritten as a Snakemake workflow as follows. Transcripts were aligned to the human genome using minimap2(18), using an alignment index built on the gencode v28 primary human genome. Sequencing errors in aligned long-reads were corrected using TranscriptClean (19) with default parameters. Splice junctions for TranscriptClean were obtained using the TranscriptClean accessory script “get\_SJs\_from\_gtf.py” using the gencode v28 comprehensive annotation as the input. A list of common variants to avoid correcting were obtained from the ENCODE portal (<https://www.encodeproject.org/files/ENCFF911UGW/>). The long-read transcriptome annotation was generated with TALON (20). A TALON database was generated using the talon\_initialize\_database command, with all default parameters, except for the “–5P” and “–3p” parameters. These parameters represent the maximum distance between close 5’ start and 3’ ends of similar transcript to merge and were both set to 100 to match parameters used in later tools. Annotation in GTF format was generated using the talon\_create\_GTF command, and transcript abundance values were generated using the talon\_abundance command.

## Analysis of short-read RPE data

             Each sample was aligned to the Gencode release 28 hg38 assembly using the genomic aligner STAR and sorted the resulting BAM files using samtools sort. (1),(19),(20). For each sorted BAM file, a a per sample base transcriptome was constructed using StringTie with the Gencode V28 comprehensive annotation as a guiding annotation (1),(11). All sample transcriptomes were merged with the long-read transcriptome using gffcompare(21) with default parameters. We note that the default values for the distance to merge similar 5’ starts and 3 ends of transcripts in gffcompare is the same to what we chose for TALON. We define the metric construction accuracy, used to evaluate short-read transcriptome construction as the following:

## Construction of tissue specific transcriptomes.

             We used studies with healthy, unperturbed RNA-seq samples from 52 distinct subtissue regions of the body, downloaded and performed quality control the pertinent sequencing data from the sequence read archive (SRA) using methods from our previous work (15). We constructed a transcriptome for each sample, and merged samples together to create 52 subtissue specific transcriptomes. For each tissue-specific transcriptome, we removed transcripts that had an average expression less than 1 Transcripts Per Million (TPM) across all samples of the same tissue type. All tissue specific transcriptomes were merged to form a single unified GTF annotation file to ensure transcript identifiers were the same across tissues. We merged all ocular tissue transcriptomes to generate a separate pan-eye transcriptome.

## Tissue specific transcriptome quantification

             For each resulting tissue specific transcriptome, we extracted transcript sequences using the tool gffread (21) and used these sequences to build a tissue-specific quantification index using the index mode of the alignment free quantification tool Salmon. (22) For each sample, we quantified transcript expression using the quant mode of salmon, using a sample’s respective tissue specific quantification index. We similarly quantified all ocular samples using the pan-eye transcriptome and the Gencode v28 reference transcriptome.

## Annotation of novel exons

             Analysis of novel transcripts was done using a custom Rscript “annotate\_and\_make\_tissue\_gtfs.R” . First, a comprehensive set of distinct, annotated exons was generated by merging exon annotation from gencode, ensembl, UCSC, and refseq. We then defined a novel exon as any exon within our transcriptomes that does not exactly match the chromosome, start, end and strand of an annotated exon. Novels exons were classified by splitting exons into 3 categories: first, last, and middle exons. We then extracted all annotated exon start and stop sites from our set of previously annotated exons. Novel middle exons that had an annotated start but an unannotated end were categorized as a novel alternative 3’ end exons and similarly novel middle exons with an unannotated start but annotated end were categorized as a novel alternative 5’ start exons. Novel middle exons whose start and end both matched annotated exon start and ends are considered retained introns. Novel middle exons whose start and end both did not match annotated starts and ends are considered fully novel exons. We then classified novel first and last exons. Novel first exons are first exons whose start is not in the set of annotated exon starts, and novel last exons are terminal exons whose end is not in the set of annotated exon ends.

## Validation of DNTX with phylop, CAGE data, and polyA signals

             PhyloP (23) scores for the phylop 20 way multi species alignment were downloaded from UCSC’s FTP server on October 16th, 2019 and converted from bigWig format to bed format using the wig2bed tool in BEDOPs (24) . The average score per exon in both the gencode and DNTX annotation was calculated by intersecting exon locations with phylop scores and then averaging the per base score for each exon, using the intersect and groupby tools from the bedtools suite, respectively. Significant difference in mean phylop score was tested with a Mann Whitney U test.

             CAGE peaks (25) were download from the FANTOM FTP server (<https://fantom.gsc.riken.jp/5/datafiles/reprocessed/hg38_latest/extra/CAGE_peaks/hg38_fair+new_CAGE_peaks_phase1and2.bed.gz>) on June 15th 2020. Transcriptional start sites (TSS) were extracted from gencode and DNTX annotations; TSS is defined as the start of the first exon of a transcript. Distance to CAGE peaks was calculated using the closest tool in the bedtools suite. Significant difference in mean distance to CAGE peak between DNTX and gencode annotation was tested with a Mann Whitney U test.

             Polyadenylation signal annotations were downloaded from the polyA site atlas (26) (<https://polyasite.unibas.ch/download/atlas/2.0/GRCh38.96/atlas.clusters.2.0.GRCh38.96.bed.gz>) on June 15th 2020. Transcriptional end sites(TES) were extracted from gencode and DNTX annotations; TES is defined as the end of the terminal exon of a transcript. Distance to polyA signal was calculated using the closest tool in the bedtools (27) suite. Significant difference in mean distance to polyA signal was tested with a Mann Whitney U test.

## Identification of protein coding novel transcripts.

             Protein coding transcripts in our unified transcriptome were identified using the TransDecoder suite (10). Transcript sequences in fasta format were extracted from the final pan-body transcriptome using the TransDecoder util script “gtf\_genome\_to\_cdna\_fasta.pl”. Potential open reading frames(ORFs) were generated from transcript sequences using the LongestORF module within TransDecoder, and the single best ORF for each transcript was extracted with the Predict module within Transdecoder. The resulting ORFs were mapped to genomic locations with the TransDecoder util script “gtf\_to\_alignment\_gff3.pl”. For each ORF start and stop codons were extracted with the script “agat\_sp\_add\_start\_stop.pl” scripts from the AGAT toolkit (<https://github.com/NBISweden/AGAT/>). Transcripts with no detectable ORF or missing a start or stop codon were labelled as noncoding.

## Analysis of novel isoforms in eye tissues.

             An Upset (28) plot was generated using the ComplexUpset package(<https://github.com/krassowski/complex-upset>). Fraction Isoform Usage(FIU) was calculated for each transcript t associated with a parent gene g using the following formula: . Raincloud plots of FIU were generated using the R\_Rainclouds package. (29)

## Prediction of variant impact using *de novo* transcriptomes.

             Noncoding variants previously associated with retinal disease from the Blueprint Genetics Retinal dystrophy panel were obtained from the Blueprint Genetics website (<https://blueprintgenetics.com/tests/panels/ophthalmology/retinal-dystrophy-panel/>). The variants were converted from HGVS to VCF format using a custom python script “HGVS\_to\_VCF.py”. This VCF was then remapped to the hg38 human genome build using the tool crossmap (30). The VCF of variants was used as the input variants for the Variant Effect Predictor(VEP) tool from Ensembl (31), with our fetal and adult retina transcriptomes as the input annotation. VEP was additionally run using the gencode V28 comprehensive annotation as the input annotation to identify variants whose predicted impact increased in severity.

## Analysis of fetal retina RNA-seq data.

             RNA-seq samples from Mellough et al. were downloaded from the SRA using methods from a previous study (15) Samples were quantified using salmon with a quantification index generated using our fetal retina *de novo* transcriptome. Outliers within the dataset were identified by first performing principal component analysis of transcript level expression data, calculating the center of all data using the first two principal components, and the 5 samples furthest away from the center of all data were removed. The remaining samples were normalized using calcNormFactors from the edgeR R package (32) and converted to weights using the voom function from the limma R package. (33) Differential expression was modeled using the lmFit function using developmental time point as the model design and tested for significant change in expression using the Ebayes function from limma. Gene Set enrichment was testing using the clusterprofileR package. (34) Heatmaps were generated using the ComplexHeatmap package (35).

## Computing Resources

             All computation was performed on the National Institutes of Health high performance compute system Biowulf (hpc.nih.gov).

## Figures and Tables

             All statistical analyses, figures and tables in this paper were generated using the R programming language. (36) A full list of packages and versions can be found in supplementary file session\_info.txt

# Results

## Long-read Pacbio RNA sequencing guides *de novo* transcriptome construction

             In order to evaluate the accuracy of short-read transcriptome construction, we first generated PacBio long-read RNA-seq data and Illumina short-read RNA-seq data from an induced pluripotent stem cell derived RPE. These cells were differentiated using a highly optimized protocol, and thus should have minimal biological variation (37), (38). We used this sequencing data to construct a long-read transcriptome and a short-read transcriptome. In our long-read transcriptome we found 1163239 distinct transcripts, and in our short-read transcriptome 366888 distinct transcripts

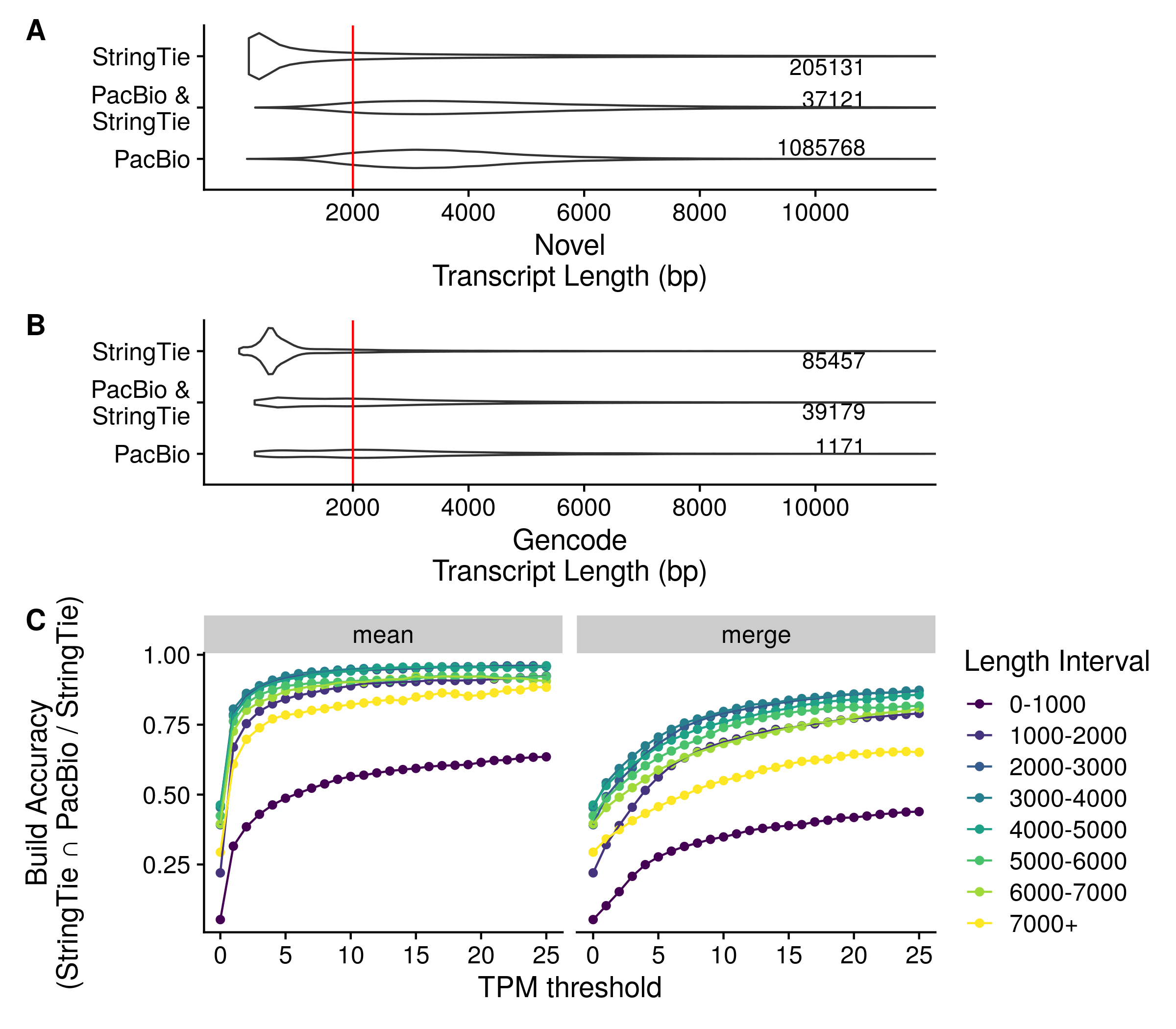


Figure 1. Transcript lengths are substantially longer in the long-read based approach. A,B) Intersection of novel and annotated transcript lengths between Pacbio (long-read) and Stringtie (short-read) transcriptomes. The total number of constructed transcripts is given in the text to the right of the violin plot. C) short-read construction accuracy stratified by transcript length at different TPM based transcript exclusion thresholds.

             In our initial comparison between short and long-read transcriptomes, we saw a low transcriptome construction accuracy (see Methods) of 0.208. When we examined the transcript lengths of each build we saw that the two methods show very different transcript length distributions for both novel and previously annotated transcripts, with the short-read build was comprised mostly of smaller transcripts (Fig 1A). As the PacBio data was generated using two different libraries for 2000 bp and >3000 bp transcripts, we expected to see enrichment for longer transcripts in the Pacbio data set. (Supplemental Fig 2) To assess accuracy relative to transcript length, we grouped transcripts by length in 1000 bp intervals, and compared accuracy between each group. We found the accuracy significantly improves for transcripts longer than 2000 bp. The construction accuracy is 0.426 and 0.137 for transcripts above and below 2000 bp, respectively.(Fig 1B)

             We experimented with various methods to remove spurious transcripts to improve construction accuracy. We first removed transcripts that were not expressed at 1 TPM in at least one sample as outlined in StringTie’s recommended protocol. (39) This improved construction accuracy to 0.475 for transcripts longer than 2000bp and 0.212 for transcripts shorter than 2000bp. As this accuracy was still fairly low, we tried different filtering schemes. We experimented with machine learning based strategies to identify transcripts that were computational artifacts (data not shown), but we found that the simplest approach with high performance was to retain transcripts that had an average TPM above a specific threshold(Fig 1C). In our downstream pipeline we keep transcripts that have at least an average of 1 TPM across all samples of the same subtissue type as this threshold achieved a build accuracy of 0.772 for transcripts longer than 2000Bp and retained 48470 transcripts within this short-read RPE dataset.

## A rigorous analysis pipeline finds thousands of novel gene isoforms

| Tissue | Source | Samples | Studies | Transcriptome Count |
| --- | --- | --- | --- | --- |
| Retina | Adult | 105 | 8 | 49714 |
| RPE | Fetal | 49 | 7 | 49967 |
| Cornea | Adult | 43 | 6 | 51469 |
| Retina | Fetal | 89 | 6 | 66255 |
| RPE | Adult | 48 | 4 | 32012 |
| Cornea | Fetal | 6 | 2 | 59408 |

Table 1. Ocular sample dataset overview and transcriptome count. Transcriptome count is defined as the number of unique transcripts expressed in a given tissue type

             We built transcriptomes from 368 published, publicly available ocular tissue RNA-seq samples curated in EiaD using an efficient Snakemake pipeline (18). We included both adult and fetal tissue from cornea, retina, and RPE tissues mined from 29 different studies (Table 1). Our fetal tissues consist of both human fetal tissues and human induced pluripotent stem cell (iPSC) derived tissue, as stem cell derived tissue has been showed to closely resemble fetal tissue (40). To more accurately determine the tissue specificity of novel ocular transcripts, we supplemented our publicly collated normal (non-disease, perturbation) ocular data set with 877 samples across 46 body locations from the GTEx project and constructed transcriptomes for each of these body locations (12). We refer to each distinct body location as a subtissue here after.

      After initial construction of transcriptomes, we found 183442 previously annotated transcripts and 6241675 novel transcripts detected in at least one of our 1245 samples. We define novel as any region of the human genome that has not been previously annotated within the Gencode, Ensembl, UCSC, and Refseq annotation databases. (1) , (41) , (42) After using the filtering methods described above, we merged all tissue specific transcriptomes into a single final transcriptome which contains 252983 distinct transcripts with 87592 previously annotated and 165391 novel transcripts, and includes 114.914831 megabases of previously unannotated genomic sequence. (Table 1) We refer to the final pan-body transcriptome as the DNTX annotation hereafter.

             We split novel transcripts into two categories: novel isoforms, which are novel variations of known genes, and novel loci, which are previously unreported, entirely novel regions of transcribed sequence.(Fig 2B) Novel isoforms are further classified by the novelty of its encoded protein: an isoform with novel open reading frame, a novel isoform with a known ORF, and isoforms with no ORF as noncoding isoforms.(Fig 2A) The number of distinct ORFs is significantly less than the number of transcripts, with 43279 previously annotated ORFs and 46226 novel ORFs across all tissues. Across all tissues there is an average of 10393 novel isoforms and 3716 novel ORFs.

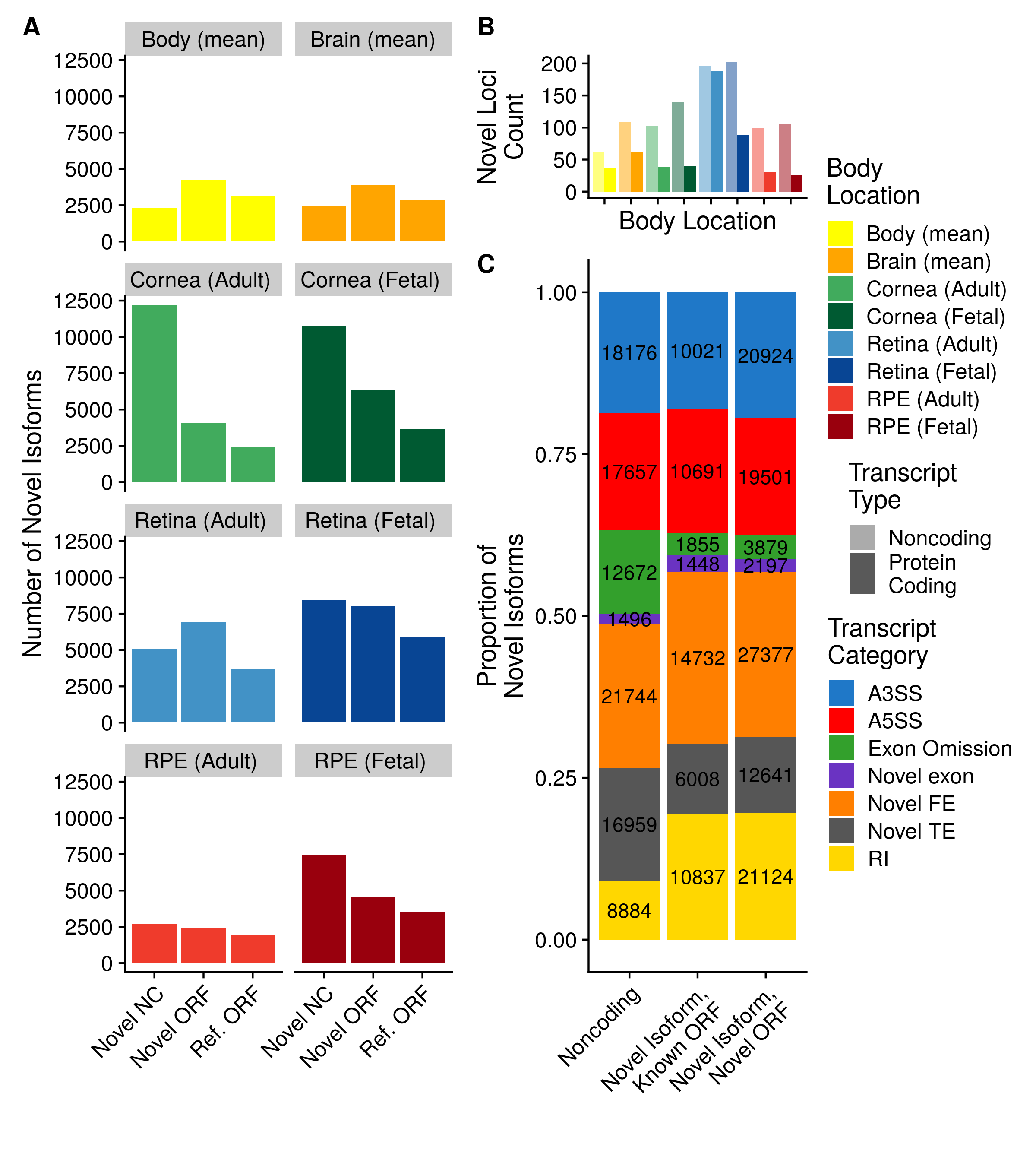


Figure 2. Overview of Novel Isoforms. A. Number of novel gene isoforms, grouped by transcript type. Brain and body represent an average of 13 and 34 distinct subtissues, respectively B. Novel protein coding and noncoding loci. Novel exon composition of novel isoforms, by isoform type labels indicate number of transcripts. C. Classification of novel exon types, stratified by novel isoform type

             Novel isoforms can occur due to an omission of a previously annotated exon, commonly referred as exon skipping or the addition of an unannotated exon which we refer to as a novel exon. We further classified novel exons by the biological process that may be driving their formations: alternative promoter usage driving the addition of novel first exons (FE) (43), alternative polyadenylation driving the addition of novel terminal exons (TE) (44) , and alternative splicing driving the formation of all novel exons that are not the first or last exon (45). We then split alternatively spliced exons into their commonly seen patterns, alternative 5’ splice site (A5SS), alternative 3’ splice site (A3SS), and retained introns (RI). Exons whose entire sequence was unannotated and is not a retained intron are fully novel exons. We note that all three of these mechanisms can lead to exon skipping, so for simplicity we grouped all novel isoforms resulting from exon skipping together. We found that the majority of novel exons with our dataset are novel FEs and TEs. We saw that the majority of RI exons lead to novel ORFs, whereas novel isoforms with omitted exons more often lead to noncoding isoforms. (Fig 2C)

## *De novo* transcriptomes match previously published experimental data than existing annotation

             We validated our *de novo* transcriptomes using three independent datasets. First, we evaluated the conservation of our transcriptomes, as conservation has been a historic marker for function. We used PhyloP 20 way species alignment (23), a measure of conservation between species, to calculate the average conservation score for each exon in our DNTX annotation, and compared that to the average conservations score for each exon in the gencode annotation. We found that on average, exons in our DNTX annotation are more conserved than exons in the gencode annotation (pvalue <2.2e-16) (Supplemental Figure 3A).

             Next, as we saw an enrichment of novel first and last exons within our data set, we decided to compare the transcriptional start sites (TSS) and transcriptional end sites(TES) within our DNTX annotation to two well established annotation databases from FANTOM and the polyA Atlas (25), (26). We compared DNTX and gencode TSS’s to CAGE-seq data from the FANTOM consortium; as CAGE-seq is optimized to detect the 5’ end of transcripts, we reasoned that it can serve as a valid ground truth set to evaluate TSS detection. (46) We calculated the absolute distance of DNTX TSS’s to CAGE peaks, and compared them to the absolute distance of gencode TSS’s to CAGE peaks. We found that on average DNTX TSS’s are closer to CAGE peaks than gencode TSS’s (pvalue <2.2e-16)(Supplemental Figure 3B). Next we evaluated TES’s using the polyA Atlas, which is comprised of polyadenylation signal annotation generated from aggregating 3’ seq data from multiple studies. As 3’-seq data is designed to accurately capture the 3’ ends of transcripts it can similarly serve as a ground truth set to evaluate the accuracy of TES’s (47). We calculated the absolute distance of DNTX TES’s to annotated polyA signals and compared them to the absolute distance of gencode TES’s to polyA signals. We found that on average DNTX TES’s are closer to annotated polyadenylation signals than gencode TSS’s (pvalue <2.2e-16) (Supplemental Figure 3C)

## *De novo* transcriptomes reduce overall transcriptome sizes

             Our transcriptomes removed on average 76.141 % of a tissue’s base transcriptome. We defined base transcriptome for a tissues as any transcript in the gencode annotation with non zero TPM in at least one sample of a given tissue type. This was a large reduction in transcriptome size and we wanted to ensure that we were not unduly throwing away data. We quantified transcript expression of our samples using Salmon, quantifying each sample twice: once using the full gencode V28 human transcript annotation, and once using its associated tissue specific transcriptome We found that despite the 76.141 % reduction in number of transcripts between the base gencode and *de novo* transcriptomes (Supplemental Figure 4A), the average salmon mapping rate increases by 2.041 % indicating that the vast majority of gene expression data is retained within our transcriptome. (Supplemental Figure 4B)

## Novel Isoforms in Ocular tissues

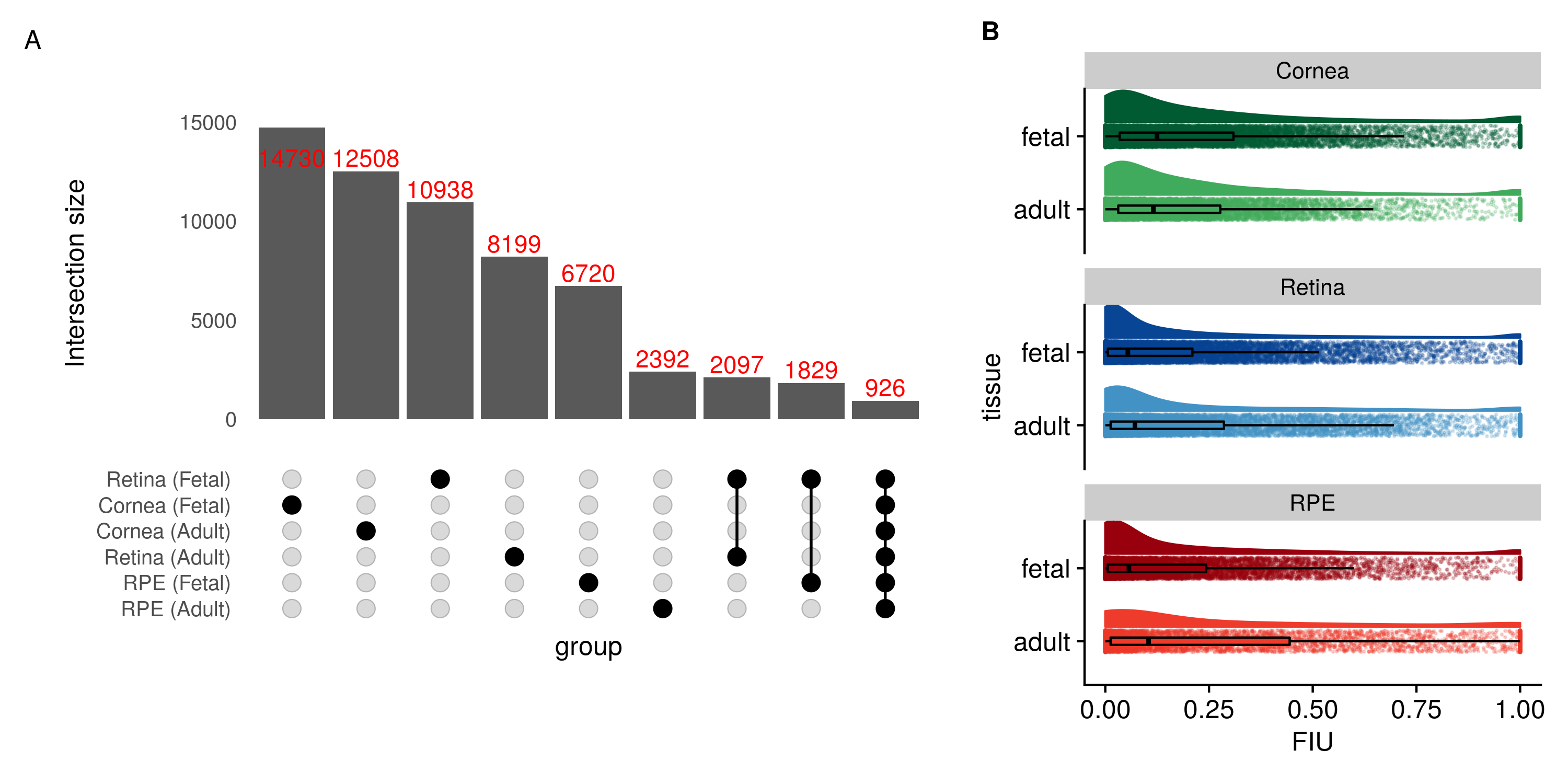


Figure 3. Overview of novel gene isoforms in the eye A). Set intersection of novel isoforms in ocular transcriptomes. B). Boxplots of fraction isoform usage(FIU) overlaid over FIU data points with estimated distribution of data set above each boxplot

             Next, we analyzed the novel isoforms within our pan-eye transcriptome. We compared the overlap in constructed novel isoforms across ocular tissues and found that 77.97 % of novel isoforms are specific to a singular ocular subtissue (Fig 3A). Additionally, fetal-like tissues have more novel isoforms that their adult counterpart. For each novel isoform we then calculated fraction isoform usage (FIU), or the fraction of total gene expression a transcript contributed to its parent gene. We found that on average novel isoforms contribute to 20.58 % of their parent gene’s expression.

## Differential Usage of Gene Isoforms Occurs during Retinal Development

             Multiple studies have shown the alternative usage of gene isoforms plays a significant role in eye development (48), (49). We hypothesized that our DNTX annotation could provide additional insight into alternative isoform usage, and identify novel gene isoforms potentially involved in eye development. We used RNA-seq data of the developing retina from Mellough et al, an independent data set that we did not include in the data used to build our transcriptomes. We used this data in combination with a subset of our DNTX annotation corresponding to fetal retina to quantify transcript expression and identify transcripts with significant changes in expression across retinal development, which we define as differential transcript usage (DTU).

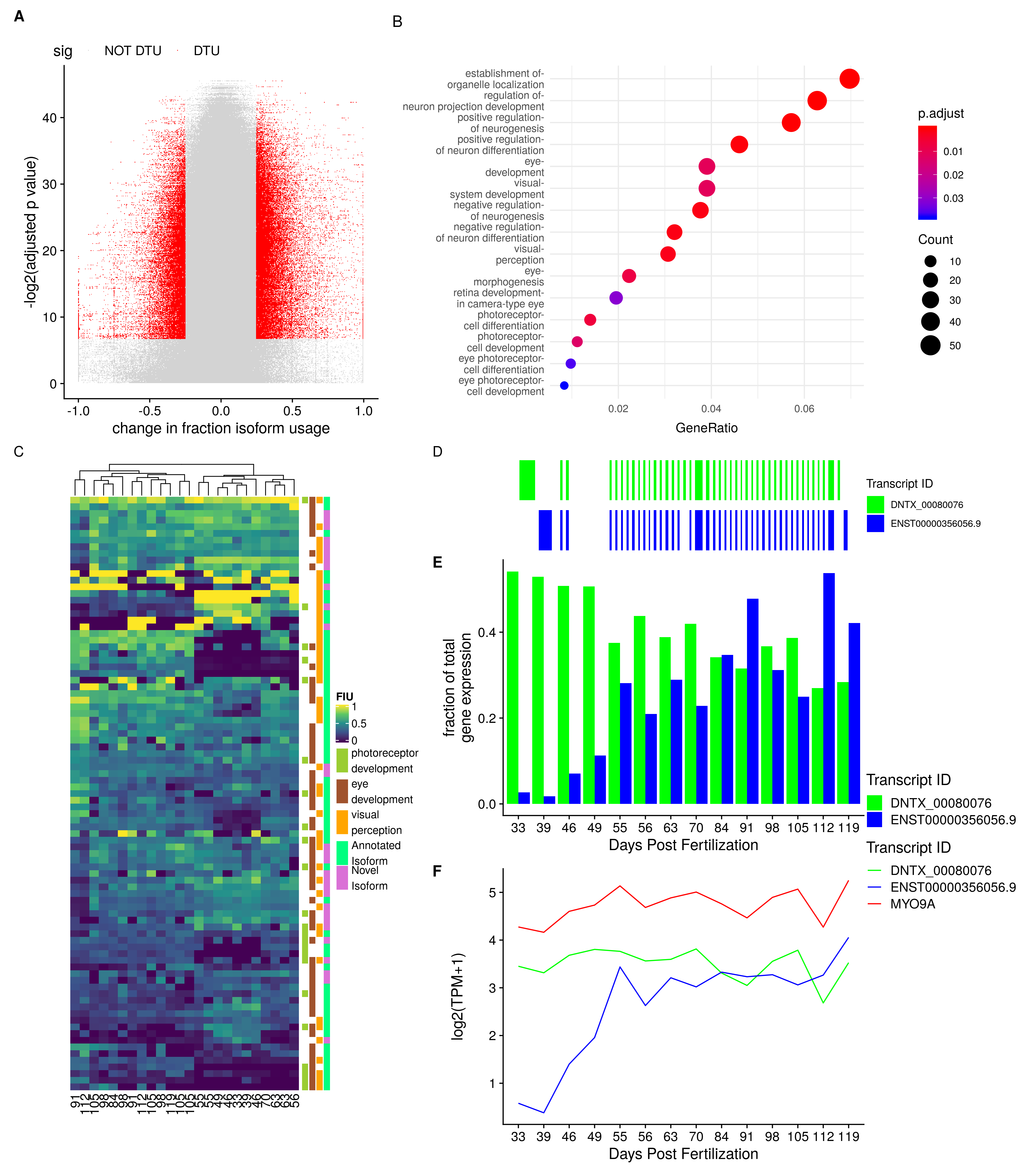


Figure 4 Differential Transcript usage during Retinal Development. A) Volcano Plot of tested transcripts B) Dot plot for gene set enrichment analysis E) Heatmap of genes with DTU associated with eye development D) Transcript models for MYO9A, a gene undergoing DTU F) change in MYO9A FIU across development F) average TPM expression of MYO9A across development

             We analyzed 24 samples across 14 developmental days post fertilization and found 1717 transcripts across 812 genes involved in differential transcript usage(DTU).(Fig 4A) We define DTU as an transcript that is differentially expressed (qvalue <.01) and has a FIU difference of .25 in at least one comparison of time points. We found that genes involved in DTU were enriched(qvalue <.05) for genes related to eye and neurological development.(Fig 4B), and that hierarchical clustering of DTU transcripts generates an early stage and late stage cluster.(Fig 4C) One of these genes, MYO9A, is a perfect example of DTU. MYO9A is associated with the visual perception GO term, plays a role in ocular development, and has been associated with ocular disease (50). While expression of MYO9A remains relatively unchanged across development, expression of two of its associated isoforms(Fig 2D) changes dramatically during development: a novel isoform is highly expressed early during development, but switches to the canonical isoform later in development (Fig 2E,F). A full list of genes and transcripts involved with DTU is available in supplemental data.

## *de novo* transcriptome allow for a more precise variant prioritization.

             The identification of a disease-causing variant through genome sequencing is a common step in diagnosing genetic disease, when disease causing variants cannot be determined from exonic variants. The prediction of the biological impact and subsequent variant prioritization is a fundamental step in this process. Many methods for predicting variant effects on protein function or gene expression are based on location within the body of a transcript; for example variants that disrupt splice sites and start/stop codons are considered to be the most damaging, while variants within intronic and intergenic regions have unknown impact or are not classified, and, thus, are not included for further consideration. However, multiple studies have identified pathogenic deep intronic variants for retinal dystrophies (51), (52), (53), (54), (55), (56), (57). Pathogenic intronic variants are thought to function by introducing a novel splice site, disrupting regulatory motifs, or altering a tissue-specific transcript. To explore this third possibility, we mapped known pathogenic intronic variants onto novel isoforms within our de novo transcriptomes.

| **Gene Name** | **Associated Disease** | **Location (hg19)** | **Canonical Variant HGVS** | **Gencode Predicted Consequence** | **DNTX Predicted Consequence** | **Published Study** |
| --- | --- | --- | --- | --- | --- | --- |
| ABCA4 | ABCA4-associated maculopathy | Chr1:94481967 C>T | c.5197–557G>T, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant | Bauwens et al. |
| Chr1:94546814 G>C | c.859–540C>G, NM\_000350.2 | intron variant | non coding transcript exon variant |
| Stargardt disease | Chr1:94484001 C>T | c.5196+1137G>A, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant | Braun et al. Zernant et al. |
| Chr1:94484082 T>G | c.5196+1056A>G, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant |
| Chr1:94526934 T>G | c.1938-619A>G, NM\_000350.2 | intron variant, splice region variant, non coding transcript variant | non coding transcript exon variant | Zernant et al. |
| Chr1:94527698 G>C | c.1937+435C>G, NM\_000350.2 | intron variant, upstream gene variant | non coding transcript exon variant | Sangermano et al. |
| Chr1:94546780 C>G | c.859-506G>C, NM\_000350.2 | intron variant | non coding transcript exon variant |
| IFT140 | Ciliopathy | Chr16:1576595 C>A | c.2577+25G>A, NM\_014714.3 | upstream gene variant, intron variant, NMD transcript variant, non coding transcript exon variant, non coding transcript variant | missense variant | Geoffroy et al. |
| PROM1 | Cone–rod dystrophy | Chr4:15989860 T>G | c.2077-521A>G, NM\_006017.2 | intron variant, upstream gene variant | 5 prime UTR variant | Mayer et al. |
| RPGRIP1 | RPGRIP1-mediated inherited retinal degeneration | Chr14:21789588 G>A | c.1611+27G>A, NM\_020366.3 | intron variant, non coding transcript variant, upstream gene variant, synonymous variant, NMD transcript variant, downstream gene variant | 5 prime UTR variant | Jamshidi et al. |

Table 2. Pathogenic variants previously considered intronic that are on expressed transcripts in the retina *de novo* transcriptome. Canonical HGVS based on cannonical

             We used a list of 129 intronic and noncoding variants previously identified as pathogenic for a retinal dystrophy and predicted the effect of these variants with Ensembl’s Variant Effect Predictor using a subset of the DNTX annotation corresponding to fetal and adult retina as the input transcript annotation. We identified ten variants whose predicted effect increased in severity due the presence of a novel gene isoform in a previously intronic region (Table 2). Seven of these variants are in deep intronic hotpsots known for pathogenic variation within the gene *ABCA4*. These variants are spanned by three distinct novel isoforms with two potentially protein coding isoforms encoding only the distal end of the canonical isoform, and one noncoding isoform encoding the upstream half the canonical isoform (Supplemental Figure 5). *ABCA4* expression and function has also been observed in RPE (58). However, we did not observe these transcripts in RPE, suggesting that these pathogenic variants are primarily affecting retinal-specific *ABCA4* transcripts. We note that these transcripts have not been experimentally validated.

             To further highlight the potential importance of our de novo transcriptomes for future genetic tests we determined how many genes associated with retinal disease have novel isoforms from RetNet(sph.uth.edu/retnet/). We found that within the set of genes with novel isoforms, there is significant enrichment of retinal disease genes (hypergeometric pvalue = 3.4e-04), with 220 out of 379 RetNet genes having a novel isoform. A full list of these genes is available in the supplementary data.

## A companion visualization tool enables easy use of *de novo* transcriptomes

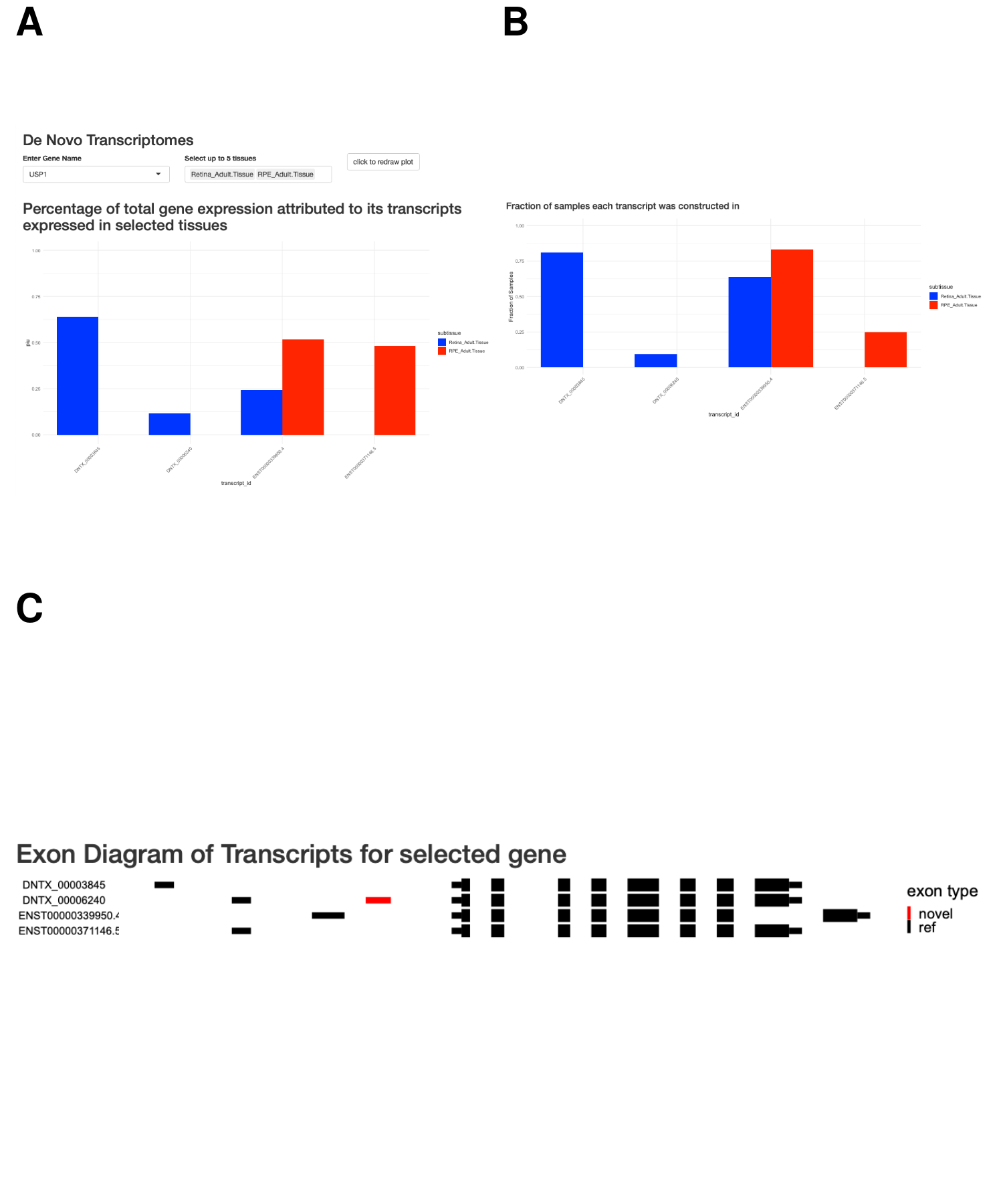


Figure 5. Screenshots from dynamic *de novo* transcriptome visualization tool. A). FIU bar plot for selected gene and tissue. B). Exon level diagram of transcript body Thicklines represent coding region of transcript. novel exons colored in red. Tooltip contains genomic location and phylop score C) Bargraph of fraction of samples within dataset each transcript was consructed in by tissue.

             To make our results easily accessible we designed a shiny app for visualizing and accessing our de novo transcriptomes. Users start by selecting a gene or searching for a gene by genomic location, and can choose up to 5 tissues to visualize transcript expression in. For each tissue we show the FIU for each transcript associated with a gene (Fig 5A). We show the exon-intron structure of each transcript and mousing over exons show genomic location overlapping SNPs, and phylogenetic conservation score (Fig 5B). We additionally show a barplot of the fraction of samples each transcript was constructed in (Fig 5C). Users can download the *de novo* transcriptomes for selected tissues in GTF and fasta format.Instructions to download and run the app are available at <https://github.com/vinay-swamy/ocular_transcriptomes_shiny>. While visualization of direct transcript expresion is not a part of this app, it can be viewed in the eyeIntegration app (15) by selected ‘DNTX’ as the transcript annotation. Finally, we package all tools used for our transcriptome pipeline within a portable docker container with a stand-alone run script. This pipeline allows other researchers to run their own samples, and generate figures and annotations similar to what is shown here, available at <https://github.com/vinay-swamy/ocular_transcriptomes_pipeline>

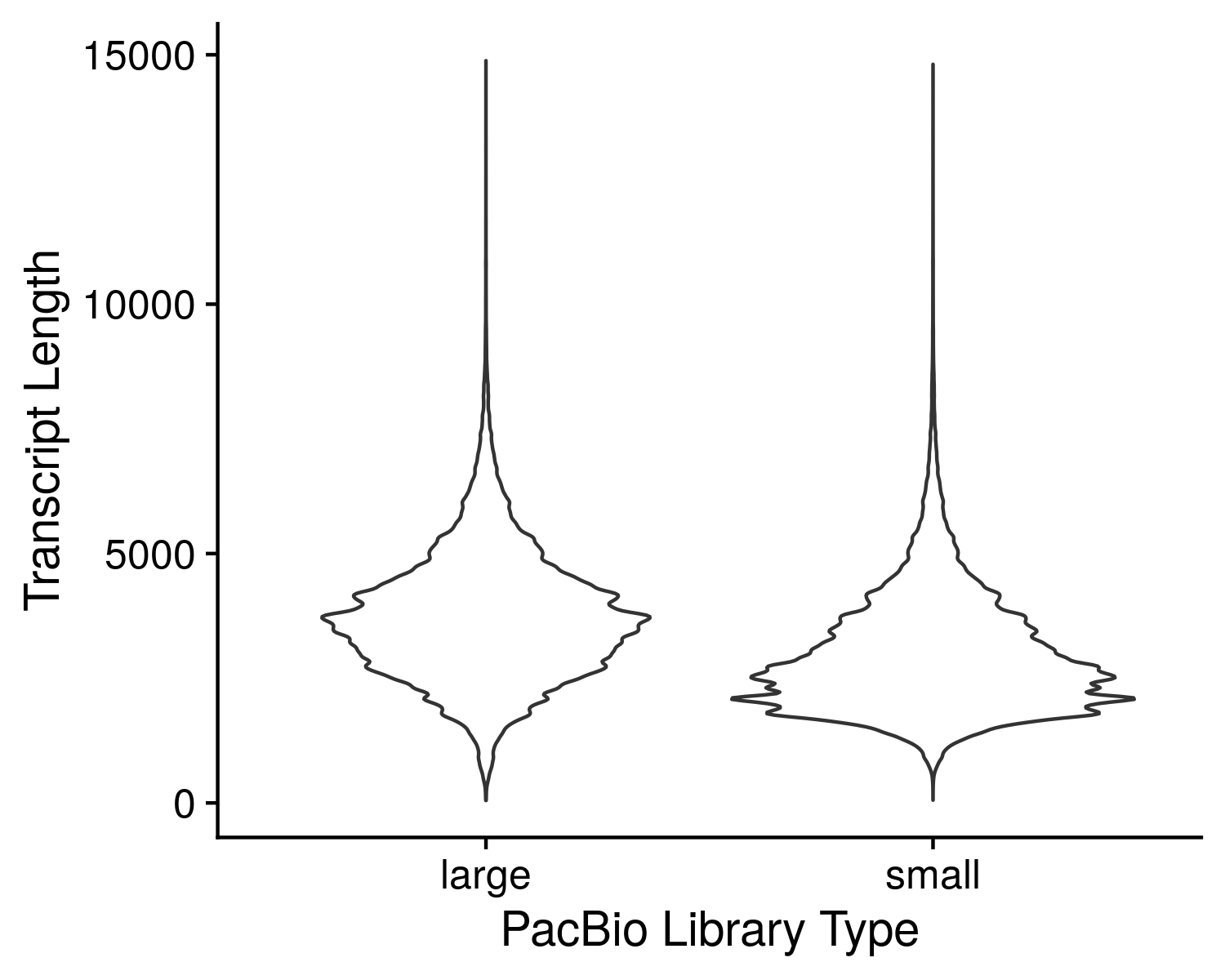
# Discussion

             Motivated by the lack of a comprehensive transcriptome for the eye, we constructed transcriptomes for adult and fetal retina, RPE and cornea. By using long-read RNA-sequencing data to calibrate our short-read construction pipeline, we were able to identify biologically relevant transcriptomes. We found that concordance between long and short-read based transcriptome is directly related to transcript length and transcript expression independently identified across many tissues. We saw a clear inability within this PacBio data set to accurately detect transcripts shorter than 2000bp for both previously annotated and novel transcripts. As many of the transcripts constructed using short-reads are below this threshold, long-read sequencing data enriched for smaller transcript sizes would provide greater insight in future studies.

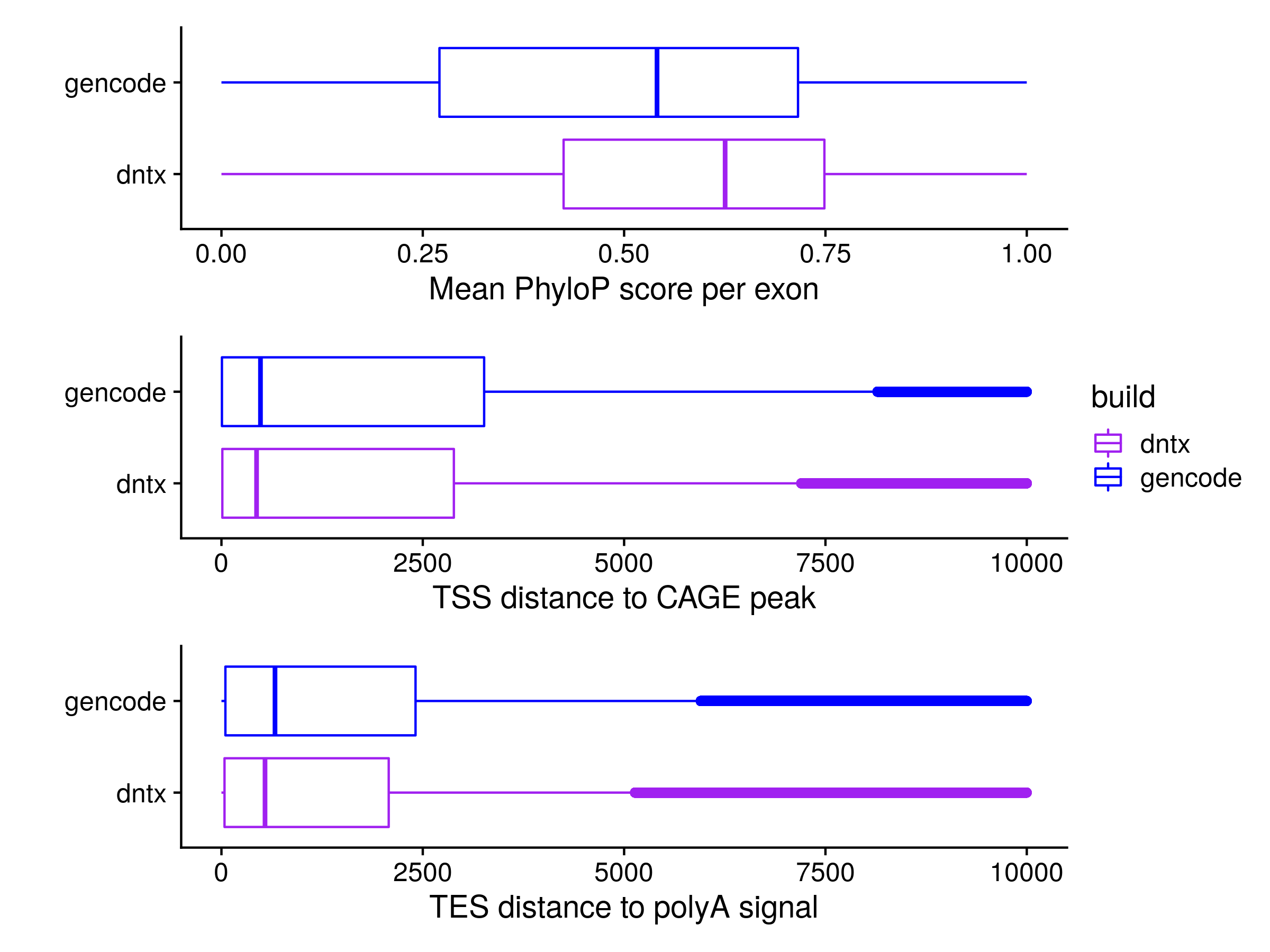
             We used a large dataset compiled from published RNA-seq data to build our ocular transcriptomes, an approach that has several key advantages. First, our large sample size allows us to combat the noisy nature of RNA-seq data. Second, as our cohort is constructed from many independent studies , we are more confident that our transcriptomes accurately reflect the biology of its originating subtissue and are not a technical artifact due to preparation of the samples. As another line of evidence, our de novo transcriptomes match existing large scale data sets and are more conserved than existing annotation. (Supplemental Figure 2)  
             In each tissue we examined, we found hundreds of novel gene isoforms, many of which were novel due to novel exons. Within ocular tissues, these novel isoforms are most commonly specific to single subtissue. This makes sense as over half of the exons in our de novo transcriptomes are first and last exons, which have been previously shown to significantly contribute to the tissue specificity of gene isoforms (59). We also found that on average novel isoforms represent about 20.58 % of their parent gene’s expression. Future studies are needed to identify the function of these isoforms. One possibility is that some of these isoforms are only expressed in rare cell types, as transcript annotation was previously shown to be incomplete in rare cell types. (60) This especially makes sense in the retina which contain over a dozen distinct cell types, several of which contribute to 5% or less of the total cell population (61). As we imposed a strict expression filter as part of our transcriptome pipeline, we may be removing transcripts specific to rare cell types.

             In conclusion, we created the first pan-eye transcriptome annotation and showed that it is useful in understanding the role of gene isoforms in ocular biology and improving the ability to diagnose inherited diseases. This work is most useful as a starting point for other researchers; we want to make our transcriptomes easily accessible to other researchers, so we designed a webapp to visualize our transcriptomes and access tissue-specific annotation files. We believe this project will enable other researchers in exploring and answering new research directions.

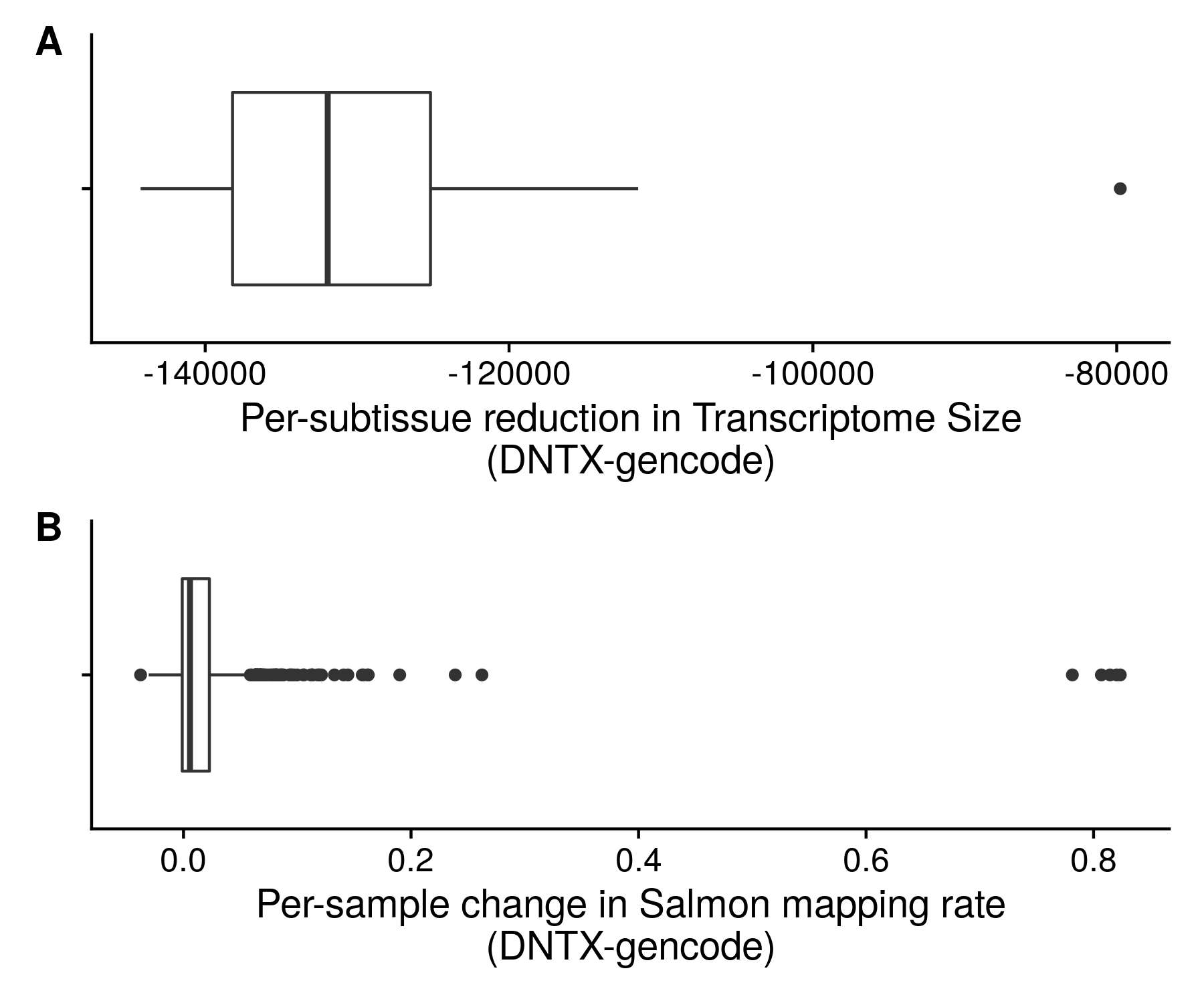
# Supplemental Figures



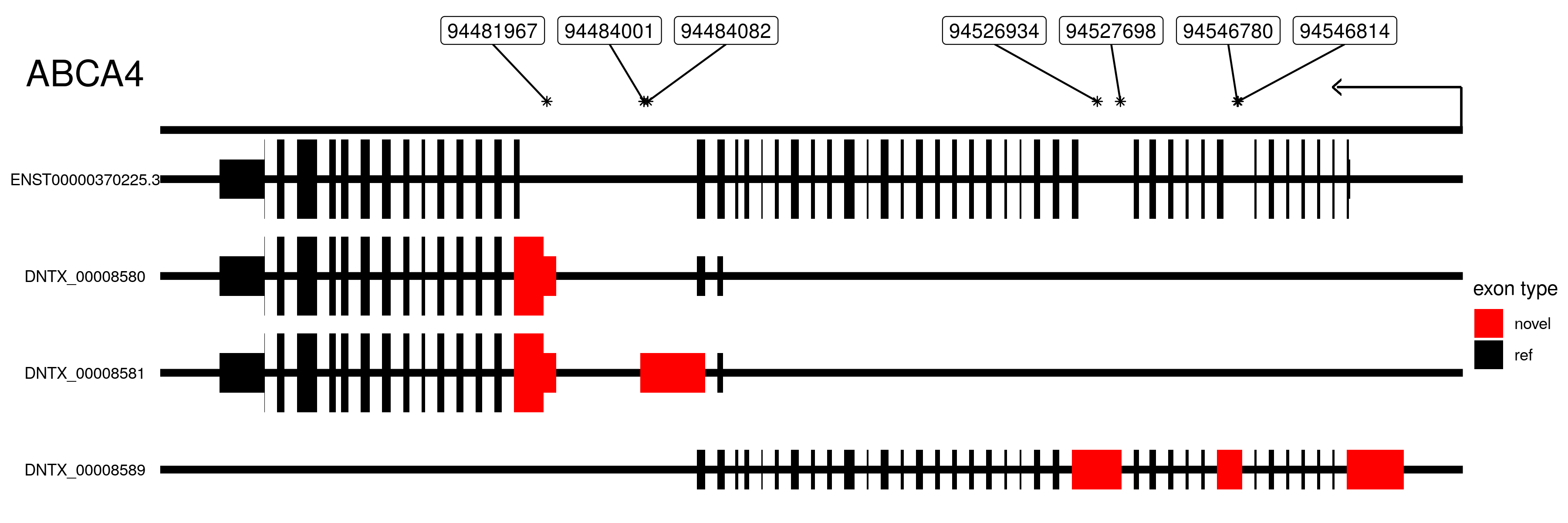
Supplemental Figure 2. Distribution of PacBio long-read lengths for two library sizes



Supplemental Figure 3. Comparison of DNTX annotation to Gencode Annotation. A) Average per exon Phylop score for gencode and DNTX transcripts. B) Average distance of DNTX Transcriptional Start Sites (TSS) and Gencode TSS to CAGE-seq peaks from the FANTOM consortium. C) Average distance of DNTX Transcriptional End Sites (TES) and Gencode TES to polyadenylation signals in the PolyA site atlas.



Supplemental Figure 4. Comparison of Salmon mapping rate change vs transcriptome size decrease.



Supplemental Figure 5. Transcript Models for selected Isoforms of ABCA4 along with location of pathogenic intronic variants. Location is on the hg19 human build. Thick line indicate protein coding region. Arrow indicates direction of transcription Introns not drawn to scale

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